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Fish skin gelatin hydrolysates produced by visceral peptidase and bovine trypsin: Bioactivity and stability

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ABSTRACT

The peptidase from the viscera of farmed giant catfish was used for producing gelatin hydrolysates (HG) and compared with those produced from commercial bovine trypsin (HB). The degree of hydrolysis (DH) observed suggests that proteolytic cleavage rapidly occurred within the first 120 min of incubation, and there was higher DH in HG than in HB. HG demonstrated the highest ACE-inhibitory activity, DPPH, ABTS radical scavenging activity, and FRAP. HB showed the highest FRAP activity. The DPPH radical scavenging activity of HG was quite stable over the pH range of 1–11, but it increased slightly when the heating duration time reached 240 min at 100 °C. The ACE-inhibitory activity of HG showed the highest stability at a pH of 7, and it remained very stable at 100 °C for over 15–240 min. The visceral peptidase from farmed giant catfish could be an alternative protease for generating protein hydrolysates with desirable bioactivities. The resulting hydrolysates showed good stability, making them potential functional ingredients for food formulations.

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1. Introduction

Enzymes of microbial origin have been widely studied and applied for producing gelatin hydrolysates with bioactivities through an enzymatic process (Aissaoui, Abidi, & Marzouki, 2015; Piyadhammaviboon, Wongngam, Benjakul, & Yongsawatdigul, 2012). However, alternative sources that are both economical and practical are in demand, and are the subject of recent studies. Currently, one of the most interesting potential alternatives is fish viscera, an under-utilized fish processing byproduct, rich in valuable substances. In recent years, self-production proteases from different types of fish viscera have been investigated and reported to be successful in producing protein hydrolysates with bioactive properties such as angiotensin I converting enzyme (ACE-I), as well as producing ACE-inhibitory and antioxidant activities (Aissaoui et al., 2015; Khantaphant, Benjakul, & Kishimura, 2011; Nasri et al., 2014).

One of the most well-known beneficial effects of bioactive peptides from gelatin hydrolysates is ACE-I inhibitory activity. Since testicular angiotensin I-converting enzyme (ACE-I, EC 3.4.15.1) is physiologically important in stimulating hypertension, the inhibition of ACE-I activity can help to control overall blood pressure. Although synthetic ACE-I inhibitors are now widely used as pro-drugs and show a remarkable effect in treating hypertension, they also cause many side effects such as dried cough, headaches, fevers, renal impairment, taste disturbances, skin rashes, insomnia, and angioneurotic edema allergic reactions (Chesiang & Sanguandeekul, 2015; Wijesekara & Kim, 2010). Therefore, food-derived compounds like protein hydrolysates/peptides are preferred for preventing hypertension to other synthetic counterparts.

Other beneficial effects of bioactive peptides include the scavenging of free radicals and reactive oxygen species that prevent oxidative damage by interrupting the radical chain reaction of lipid peroxidation. However, these kinds of free radicals and reactive oxygen species can also cause severe diseases, such as arteriosclerotic vascular disease and cancer, and they can deteriorate food rapidly (Kris-etherton et al., 2002). Therefore, there is significant concern about natural antioxidants, especially peptides derived from food protein hydrolysis due to potential health risks.

To maximally utilize the by-products during fish processing, peptidase can be extracted from the fish viscera and gelatin from the skin. Moreover, the protease from fish by-products is cost effective and could be a cheaper alternative for food enzyme applications. The present investigation is designed to produce gelatin protein hydrolysates from the skin of giant catfish through the hydrolysis by the same fish visceral peptidase compared to commercial bovine pancreas trypsin. The bioactivities, including

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ACE-inhibitory and antioxidant activities of the resulting gelatin hydrolysate, were determined. The stability of the hydrolysate at different pHs and temperatures, as well as after digestion in the gastrointestinal tract model system, was also investigated.

2. Materials and methods

2.1. Chemicals

Hippuryl-Lhistidyl-L-leucine (HHL), angiotensin converting enzyme (ACE) from rabbit lung, 2,4,6-trinitrobenzenesulfonic acid solution (TNBS), 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid monosodium salt hydrate (Ferrozine®), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), trypsin from bovine pancreas (EC 3.4.21.4), pancreatin from porcine pancreas, bile extract porcine, and pepsin from porcine gastric mucosa (EC 3.4.23.1) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin from bovine pancreas (CAS NO. 9002-07-07, 3312.2 U/mg), tri-chloroacetic acid (TCA), hydrochloric acid, sodium hydroxide, tris-(hydroxymethyl)-aminomethane) were obtained from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), iron (II) sulfateheptahydrate ($FeSO_4{\cdot}7H_2O$), calcium chloride ($CaCl_2$), and potassium persulfate ($K_2S_2O_8$) and other chemicals with analytical grade were purchased from UNIVAR, (Ajax Finechem, Sydney, Australia).

2.2. Preparation of crude enzyme extract

The pool viscera of a 3-year-old farmed Mekong giant catfish (*Pangasinodon gigas*) was used as the starting material for crude enzyme extraction. The viscera was cut into small pieces and homogenized with an extraction buffer (10 mM Tris-HCl pH 8.0, containing 10 mM $CaCl_2$) (1:5, w/v) for 2 min. The mixture was centrifuged at 10,000×*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was collected and referred to as the "crude enzyme extract" (CE). The protein concentration and protease activity in the CE was measured before performing extraction of visceral peptidase.

The peptidase from the viscera was prepared with an aqueous two phase (ATPS) method as described in Ketnawa, Martinez-Alvarez, Benjakul, and Rawdkuen (2015). The ATPS was prepared in 50 ml-graduated centrifuge tubes. 70% CE (w/w) was weighed and added to a system consisting of 15% PEG2000-15% sodium citrate. The system was mixed thoroughly for 15 min using a Vortex mixer. Phase separation was achieved by centrifuge at 4000×*g* for 10 min at 4 °C. To form the 2nd ATPS cycle, another 10% (w/w) sodium citrate was added to the top phase of the 1st ATPS cycle previously separated. The upper phase from the 2nd ATPS was subjected to dialysis in distilled water for 6 h at 4 °C with 3 intervals of changing water. After dialysis, the obtained peptidase was subjected to protein content and protease activity analysis before further use in gelatin hydrolysis. The protein content and enzyme activity of visceral peptidase after ATPS partitioning was measured by using BAPNA as a substrate as described in Bradford (1976) and in Benjakul, Visessanguan, and Thummaratwasik (2000), respectively. The obtained fraction was investigated for protein patterns and zymography, which revealed different bands that corresponded with different alkaline peptidases. These also included a trypsin-like enzyme (data not shown). The enzyme used in the experiments was referred to as the visceral peptidase and abbreviated as G.

2.3. Fish skin gelatin preparation

Gelatin was extracted from the prepared fish skin of farmed giant catfish as described in Sai-Ut, Jongjareonrak, and Rawdkeun (2010). NaOH (0.2 M) was used for soaking the skin (skin: solution ratio of 1:10, w/v) at 4 ± 1 °C for 2 h with continuous gentle stirring. Then, the alkaline-treated skin was washed with tap water until the washed water reached pH < 7.5. The washed skin was soaked in 0.05 M acetic acid (skin: solution ratio of 1:10, w/v) for 3 h at room temperature (25 ± 1 °C). The acid-treated skin was washed as previously described. The fish skin subsequently swelled and was soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45 ± 1 °C for 12 h with continuous stirring to extract the gelatin. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was lyophilized and the resulting dry matter was ground and referred to as "gelatin powder".

2.4. Preparation of gelatin hydrolysates

The gelatin (1 g) from the giant catfish skin, which had 85.27% (dry basis) protein content, was mixed with 100 ml of 50 mM Tris-HCl buffer with a pH of 8.0 to obtain a final protein concentration of 10 mg/ml. The gelatin solution was adjusted to the required pH of each enzyme with 1 M NaOH or 1 M HCl and then was pre-incubated for 5 min at 70 °C and 55 °C for both hydrolysis by G and commercial bovine trypsin (B), respectively. To start the hydrolysis, different levels of enzymes (2.0, 4.0, 6.0, 8.0 and 10.0 Units) were added into the gelatin solution in the ratio of E/S 1:1. After 2–12 h of hydrolysis, the enzymes were deactivated by boiling in a temperature controlled water bath for 10 min (W350, Memmert, Schwabach, Germany). Thereafter, the mixture was centrifuged at 8000×*g* at room temperature (23–25 °C) for 10 min. For the control sample, the hydrolysate was obtained by using the same procedure, and distilled water was used instead of the enzyme solution. The supernatant was collected and referred to as the gelatin hydrolysates (HG) and the commercial bovine pancreas trypsin (HB). All gelatin hydrolysates were measured for protein content by the Biuret method (Gornall & Bardawill, 1949) and the properties were determined at a protein concentration of 5 mg/ml.

2.5. Properties of gelatin hydrolysates determinations

2.5.1. The degree of hydrolysis (DH) determination

The DH of the gelatin hydrolysates was analyzed according to the method of Benjakul and Morrissey (1997). Two millilitres of 0.2 M phosphate buffer with a pH of 8.2 and 1.0 ml of 0.01% TNBS solution were added to the samples (125 µl). The solution was mixed completely, shaded from light, and placed in a water bath at 50 °C for 30 min. Sodium sulfite (0.1 M) was added to terminate the reaction. The mixtures were cooled for 15 min at room temperature. The absorbance was measured at 420 nm and α-amino acid content was expressed in terms of L-leucine. The DH was calculated as follows:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

L_t is the amount of α-amino acid released at time t. L_0 is the amount of α-amino acid in the original gelatin solution. L_{max} is the total α-amino acid in the original gelatin solution obtained after acid hydrolysis with 6 N HCl at 100 °C for 24 h.

2.5.2. Measurement of angiotensin I-converting enzyme (ACE-I)-inhibitory activity

The ACE-I inhibitory activity of the gelatin hydrolysates was determined as described in Hayakari, Kondo, and Izumi (1978) with slight modifications. The sample (0.3 ml) was incubated with

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